

## CONCENTRATION OF FATTY ACID ETHYL ESTERS IN HAIR OF ALCOHOLICS: COMPARISON TO OTHER BIOLOGICAL STATE MARKERS AND SELF REPORTED-ETHANOL INTAKE

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**Abstract — Aims:** In a variety of clinical and forensic situations long term use of alcohol must be monitored. In this project we explore the utility of fatty acid ethyl esters (FAEE) in this regard. Additionally, we propose a cut-off value of FAEE to distinguish teetotallers/moderate/social drinkers from alcoholics or individuals drinking at harmful levels. **Patients and methods:** FAEE levels from 18 alcohol-dependent patients in detoxification were contrasted with those of 10 social drinkers and 10 teetotallers. FAEE in hair were determined, using headspace solid phase microextraction and gas chromatography mass spectrometry.  $C_{FAEE}$ , as sum of the concentrations of four esters, was compared to a major FAEE, ethyl palmitate. PEth was measured in heparinized whole blood with a high pressure liquid chromatography (HPLC) method. Drinking validation criteria include self reports, phosphatidyl ethanol (PEth) in whole blood as well as the traditional markers of heavy drinking, gamma glutamyl transpeptidase (GGT), mean corpuscular volume (MCV) and carbohydrate deficient transferrin (CDT). **Results:** Receiver-operating characteristic (ROC) curve analysis for  $C_{FAEE}$  indicated a sensitivity of 100% and a specificity of 90% for a cut-off of 0.29 ng/mg. By using a cut-off of 0.4 ng/mg,  $C_{FAEE}$  identified 94.4% correctly.  $C_{FAEE}$  and ethyl palmitate were significantly associated ( $r = 0.945$ ;  $P < 0.001$ ) as were  $C_{FAEE}$  and PEth ( $r = 0.527$ ;  $P = 0.025$ ). No significant correlation was found between  $C_{FAEE}$  and total grams of ethanol consumed last month, blood-alcohol concentration at admission to the hospital, CDT, MCV, or GGT. Among the serum and blood markers, %CDT identified 47.1%, MCV 38.8% and GGT 72.2% of patients with chronic intake of higher amounts of ethanol correctly, whereas PEth achieved 100% accuracy. **Conclusions:** The data suggest that  $C_{FAEE}$  is a potentially valuable marker of chronic intake of high quantities of ethanol. Furthermore, the results indicate that a reasonable and provisional FAEE cut-off to distinguish between social/moderate and heavy drinking/alcoholism in hair is 0.4 ng/mg.

### INTRODUCTION

The global burden of disease from alcohol exceeds that of tobacco and is on a par with the burden attributable to unsafe sex practices world-wide (World Health Organization, 1999). Accurate self report strategies, biological state markers, combinations of alternative biomarkers, and combinations of biomarkers and self reports capable of monitoring alcohol consumption with a high sensitivity and specificity over the entire time spectrum are needed.

Especially during the last decade, three non-oxidative direct ethanol metabolites have attracted attention. Promising markers of this type include fatty acid ethyl esters (FAEE) (Wada *et al.*, 1971; Doyle *et al.*, 1994, 1996; Dan and Laposata, 1997; Diczfalusy *et al.*, 1999, 2001), ethyl glucuronide (EtG) (Schmitt *et al.*, 1995; Alt *et al.*, 2000; Seidl *et al.*, 2001; Wurst *et al.* 1999a,b, 2000, 2002, 2003), and phosphatidyl ethanol (PEth) (Alling *et al.*, 1983, 1984; Hansson *et al.*, 1997; Gunnarsson *et al.*, 1998; Varga *et al.*, 1998, 2000). Each of these remains positive in serum and urine for a characteristic time spectrum after cessation of alcohol intake: FAEE in serum up to 24 h, EtG in urine up to 5 days, PEth in whole blood more than 2 weeks.

Recent studies have supported the use of phosphatidyl ethanol (PEth) in blood as a marker of alcohol misuse. Chronic alcohol-dependent patients admitted for detoxification had mean PEth levels of 13.2  $\mu\text{mol/l}$  on the first day, and levels remained detectable up to 14 days after admission (Hansson *et al.*, 1997). Using liquid chromatography electrospray mass spectrometric detection (HPLC–ELSD), PEth has been detected in extracts of blood from alcoholics (Gunnarsson *et al.*, 1998). These patients had PEth levels of 5–13  $\mu\text{mol/l}$ , which remained detectable up to 3 weeks after the beginning of an alcohol-free period. A third study on chronic alcoholics showed mean PEth levels of 2.5 and 5.1  $\mu\text{mol/l}$  in two different groups respectively (Varga *et al.*, 2000). A study on healthy volunteers revealed that a single dose of ethanol (32–47 g) does not produce measurable amounts of PEth (Varga *et al.*, 1998). However, out of twelve volunteers who consumed between 624 and 2134 g of ethanol during a 3 week period, eight persons produced levels of PEth of 1.0–2.1  $\mu\text{mol/l}$ . A threshold of total ethanol intake yielding detectable PEth seems to be around 1000 g, with a mean daily intake of about 50 g. Thus far, analysis of PEth has been performed using whole blood, however, a recent study on blood from chronic alcoholics showed that almost all PEth was found in the erythrocyte fraction (Varga *et al.*, 2000).

Fatty acid esters of ethanol (FAEE) have been implicated as possible mediators for at least some of the toxic effects associated with alcohol consumption, as short term markers of

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ethanol intake in serum and as long term markers in hair. Direct toxic effects of FAEE have not been demonstrated (Laposata and Lange, 1986; Doyle *et al.*, 1996; Dan and Laposata, 1997; Soderberg *et al.*, 1999), although these esters have been shown to uncouple mitochondrial oxidative phosphorylation in rabbit heart (Lange and Sobel, 1983), inhibit protein synthesis and cell proliferation in human hepatoblastoma cells (Szczypiorski *et al.*, 1995) and render pancreatic lysosomes more fragile (Haber *et al.*, 1993).

Two enzymatic activities catalyse the formation of FAEE: acyl-coenzyme A:ethanol *O*-acyltransferase (AEAT) and fatty acid ethyl ester (FAEE) synthases. FAEE synthases have been purified from several sources, including rabbit myocardium, human brain and rat adipose tissue, and two of these FAEE synthases were found to be identical to rat liver carboxylesterase (Tsujita and Okuda, 1992; Bora *et al.*, 1996). Furthermore, a variety of other enzymes (e.g. pancreatic lipase, lipoprotein lipase and glutathione transferases) reveal FAEE synthase activity (Tsujita and Okuda, 1992; Bora *et al.*, 1989; Riley *et al.*, 1990; Tsujita and Okuda, 1994; Chang *et al.*, 1997). At present there is no evidence for the existence of a specific FAEE synthase and the formation of FAEE from free fatty acids seems to result from the interplay of a host of enzymes and other functions. The enzyme responsible for most of the AEAT activity in rat liver is localized at the luminal side of the endoplasmic reticulum (Polokoff and Bell, 1978), but has not yet been identified or purified. Characterization of AEAT and FAEE synthase activities using isolated rat liver microsomes and human tissue homogenates suggests that AEAT is quantitatively the more important of these two activities (Diczfalusy *et al.*, 1999, 2001).

FAEE have also been suggested as markers of ethanol intake. In serum and erythrocytes, FAEE can be detected up to 24 h after drinking. They are not stable in blood samples due to continued enzyme activity, but are incorporated into hair and can be analysed in this medium even after several months (Pragst *et al.*, 2001). In these investigations, the sum of the concentrations ( $C_{\text{FAEE}}$ ) of the four esters: ethyl myristate, ethyl palmitate, ethyl oleate and ethyl stearate, was used as an alcohol intake marker. The incorporation was found to occur mainly from sebum into the completed hair, which results in an increase of  $C_{\text{FAEE}}$  by accumulation from proximal to distal hair sections (Auwärter *et al.*, 2001). Beside the effects of inter-individual differences in the sebum production, variables such as the kind and frequency of hair care and hair cosmetics can influence the efficiency of deposition (Hartwig *et al.*, 2003a). The differences in  $C_{\text{FAEE}}$  between hair samples from different sites of the same individual can be explained in the same way (Hartwig *et al.*, 2003b).

Based on earlier investigation of hair samples from fatalities with known excessive alcohol consumption, patients in withdrawal treatment, social drinkers and teetotallers, a cut-off value for  $C_{\text{FAEE}}$  of 1.0 ng/mg has been suggested to establish heavy alcohol use (Auwärter *et al.*, 2001; Hartwig *et al.*, 2003b).

Our aim in this project is to further evaluate FAEE in hair as an indicator of chronic intake of high amounts of ethanol and to consider the effect of using a lower cut-off to identify heavy drinking/alcohol dependence by comparing FAEE in hair with self reports, PEth in whole blood and traditional markers (GGT, MCV, CDT).

## SUBJECTS AND METHODS

### Patients

A total of 18 detoxification inpatients (14 male; 4 female) meeting ICD 10 criteria of alcohol-dependence were included. They had the following characteristics (median, SD and range is given): age 44 (SD 9.5; 24–55) years, blood alcohol concentration at admission to hospital 1.82 mg/dl (SD 0.73; 0.05–2.92), BMI 21.7 (SD 5.04; 16.6–37.6), grams of ethanol consumed last month 4440 (SD 2039; 960–7600), age of onset of disorder 22 (SD 10.3; 15–46) years, duration of disorder 16 (SD 7.17; 2–27) years, number of previous detoxifications 4 (SD 8.1; 0–30), number of cigarettes smoked per day 20 (SD 12.6; 0–50).

Data on alcohol use were obtained by using the timeline follow back procedure. The alcohol intake of the last month was reported to be representative for the last half year. Blood samples were taken at day 1 and hair samples at day 7 of hospitalization.

For comparison, previously published data from 10 social drinkers and 10 teetotallers (Pragst *et al.*, 2001) were employed. The comparison sample included seven male and 13 female subjects with a mean age of 32.9 (SD 17.9) years. The self reported ethanol consumption in grams per day was 52 (mean, SD 12.3, mean 60) for the social drinkers and zero for the teetotallers. For the teetotallers,  $C_{\text{FAEE}}$  was 0.0 mean (SD 0.0, median 0.0) and for the social drinkers 0.21 mean (SD 0.1, mean 0.7) [ng/mg]. In the Kruskal Wallis test the three groups (teetotallers, social drinkers, alcoholics) were significantly different ( $P < 0.0001$ ).

The study was reviewed by the Bavarian Chamber of Physicians Ethical Committee and all patients gave written informed consent.

### Methods

**FAEE in hair.** The analytical determination of the four FAEE was performed by external decontamination of the hair with *n*-heptane, liquid extraction with a dimethylsulphoxide/*n*-heptane mixture, separation and evaporation of the *n*-heptane layer, solid phase micro-extraction of the residue and gas chromatography-mass spectrometry using deuterated standards of all four esters. The experimental details, limits of detection, assay reproducibility and standard deviations have been described elsewhere (Pragst *et al.*, 2001; Hartwig *et al.*, 2003b).

**Phosphatidyl ethanol in whole blood.** PEth was measured in heparinized whole blood as described elsewhere by high pressure liquid chromatography (HPLC) combined with an evaporative light-scattering detector (ELSD) method (Varga *et al.*, 2000).

**%CDT in serum.** CDT estimation was performed on duplicate serum samples using the %CDT turbidimetric immunoassay kit (Bio-Rad Laboratories, Philadelphia, PA, USA) according to the manufacturer's instructions. This method is based on micro anion-exchange chromatography followed by turbidimetric measurement. Isoelectric focusing to exclude rare genetic D-variants was undertaken as previously described followed by semi-quantitative evaluation by means of a scanner (Bean and James, 1994; Kuchheuser *et al.*, 1995).

**Cut-off values.** The cut-off of 1.0 ng/mg for  $C_{\text{FAEE}}$  was compared with 0.4 ng/mg, as suggested by data from several studies and from ROC curve analysis. In addition, the conventional cut-off (0.4 ng/mg) for ethyl palmitate, which

Table 1. Descriptive statistics of parameters determined in alcohol detoxification patients

Expression	MCV[fL]	GGT [U/L]	C <sub>FAEE</sub> [ng/mg]	Ethyl palmitate [ng/mg]	PEth [nmol/L]
Median	95.3	50.5	1.09	0.46	3.7
SD	4.86	98.8	0.67	0.289	2.35
Range	89.2–107.4	12–316	0.39–3.19	0.09–1.26	0.63–8.68

Reference values: MCV <98 fL; GGT: <20 U/L for females; <28 U/L for males; C<sub>FAEE</sub> 1.0 ng/mg; ethyl palmitate 0.4 ng/mg; phosphatidylethanol (PEth) <limit of detection (LOD). MCV (mean corpuscular volume); GGT ( $\gamma$ -glutamyl transferase); C<sub>FAEE</sub> (concentration of fatty acid ethyl esters); PEth (phosphatidylethanol).

Table 2. Hair length, concentrations of the four esters ethyl myristate, ethyl palmitate, ethyl oleate, ethyl stearate and the sum of their concentrations (C<sub>FAEE</sub>) in alcohol detoxification patients

Case	Hair Length Determined (cm)	Ethyl myristate [ng/mg]	Ethyl palmitate [ng/mg]	Ethyl oleate [ng/mg]	Ethyl stearate [ng/mg]	C <sub>FAEE</sub> [ng/mg]
1	0–6	0.20	0.89	0.71	0.26	2.06
2	0–6	0.47	1.26	1.26	0.20	3.19
3	0–6	0.11	0.68	0.76	0.16	1.71
4	0–5.5	0.08	0.49	0.97	0.15	1.69
5	0–4	0.07	0.46	0.56	0.13	1.22
6	0–1.5	0.02	0.27	0.35	0.09	0.73
7	0–4	0.01	0.33	0.41	0.09	0.84
8	0–6	0.01	0.22	0.47	0.20	0.90
9	0–6	0.02	0.09	0.18	0.10	0.39
10	0–6	0.09	0.36	0.51	0.11	1.07
11	0–4.5	0.08	0.66	0.60	0.11	1.45
12	0–6	0.05	0.53	1.12	0.21	1.91
13	0–2.5	0.04	0.53	0.38	0.16	1.11
14	0–3.5	0.04	0.34	0.48	0.18	1.04
15	0–5	0.12	0.70	0.72	0.21	1.75
16	0–5	0.03	0.10	0.33	0.07	0.53
17	0–3	0.01	0.26	0.45	0.12	0.84
18	0–4.5	0.03	0.47	0.44	0.12	1.06

usually accounts for about 40% of C<sub>FAEE</sub>, was compared with a cut-off of 0.16 ng/mg.

#### Statistical analysis

For statistical analysis (descriptive statistics, Spearman rank correlation, Kruskal Wallis test) SPSS 11 was used (SPSS Inc., Chicago, IL).

## RESULTS

The following biomarker median values were found for the alcohol detoxification patients (Table 1): C<sub>FAEE</sub> 1.09 ng/mg, ethyl palmitate 0.46 ng/mg, GGT 50.5 U/L, MCV 95.3 fL, and PEth 3.7 nmol/L. Detailed information regarding hair length, ethyl myristate, ethyl palmitate, ethyl oleate, ethyl stearate and C<sub>FAEE</sub> is given in Table 2.

Receiver-operating characteristic (ROC) curve analysis (Fig. 1) indicated a sensitivity of 100% and a sensitivity of 90% for a cut-off of 0.29 ng/mg and a sensitivity of 94.4% and specificity of 90% for a cut-off of 0.46 ng/mg. Use of a cut-off value of 0.4 ng/mg on C<sub>FAEE</sub> identified 94.4% and a cut-off of 0.7 ng/mg resulted in 88.9% correct identifications. In contrast, only 55.6% were found with the cut-off of 1.0 ng/mg. The area under the curve (AUC) to distinguish between teetotallers/moderate/social drinkers and harmful drinking/alcohol dependence by comparing FAEE in hair is 0.983.

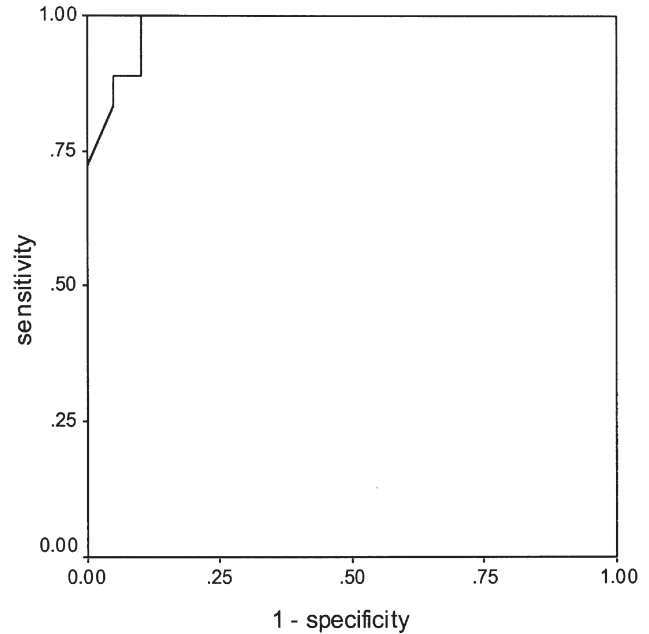


Fig. 1. ROC curve analysis for C<sub>FAEE</sub> to distinguish between teetotallers/social drinkers and heavy drinkers/alcoholics. Area under the curve (AUC) = 0.983.

Table 3. Comparison of self reported ethanol consumption and true positive results for different biomarkers in alcohol detoxification patients

Subject no.	Sex	EtOH Last month [g]	C <sub>FAEE</sub> 1 [ng/mg] Cut-off:	C <sub>FAEE</sub> 0.4 [ng/mg] Cut-off:	Ethyl Palmitate Cut-off: 0.4 [ng/mg]	Ethyl Palmitate Cut-off: 0.16 [ng/mg]	CDT	MCV	GGT	PEth (ref. value: <LOD)
1	M	7280	Pos	Pos	Pos	Pos	—	—	Pos	Pos
2	F	5600	Pos	Pos	Pos	Pos	—	—	—	Pos
3	F	7600	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
4	M	5600	Pos	Pos	Pos	Pos	—	—	Pos	Pos
5	M	3200	Pos	Pos	Pos	Pos	Pos	—	—	Pos
6	M	3200	—	Pos	—	Pos	—	—	—	Pos
7	M	7360	—	Pos	—	Pos	—	—	Pos	Pos
8	F	6400	—	Pos	—	Pos	—	Pos	Pos	Pos
9	M	4400	—	—	—	—	—	Pos	—	Pos
10	F	3800	Pos	Pos	—	Pos	—	—	Pos	Pos
11	M	4480	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
12	M	5600	Pos	Pos	Pos	Pos	Pos	—	Pos	Pos
13	M	1120	Pos	Pos	Pos	Pos	md	Pos	Pos	Pos
14	M	3360	Pos	Pos	—	Pos	—	Pos	Pos	Pos
15	M	4000	Pos	Pos	Pos	Pos	Pos	—	Pos	Pos
16	M	5280	—	Pos	—	—	Pos	Pos	Pos	Pos
17	M	1680	—	Pos	—	Pos	Pos	—	Pos	Pos
18	M	960	Pos	Pos	Pos	Pos	Pos	—	—	Pos
			12/18 55.6%	17/18 94.4%	10/18 55.6%	16/18 88.9%	8/17 47.1%	7/18 38.8%	13/18 72.2%	18/18 100%

Pos, exceeding the cut-off; —, below the cut-off; md, missing data; reference values: %CDT, <6%, MCV, < 98 fl; GGT, < 20 U/L for females; <28 U/L for males; C<sub>FAEE</sub>, 1.0 ng/mg; ethyl palmitate 0.4 ng/mg; phosphatidyl ethanol (PEth) < limit of detection (LOD).



Ethyl palmitate performed with 55.6% for the higher (0.4 ng/mg) and 88.9% with the lower cut-off (0.16 ng/mg). Table 3 gives a synopsis of the results for the blood/serum markers as well as  $C_{FAEE}$  and ethyl palmitate with different cut-offs. Among the serum/blood markers, %CDT identified 47.1%, MCV 38.8% and GGT 72.2% of the alcoholics correctly, whereas PEth reached 100%.

A significant Spearman rank correlation was found between  $C_{FAEE}$  and ethyl palmitate ( $r = 0.945$ ;  $P < 0.001$ ) and  $C_{FAEE}$  and PEth ( $r = 0.527$ ;  $P = 0.025$ ).

Correlations were not significant between  $C_{FAEE}$  and total grams of ethanol consumed last month, blood-alcohol concentration at admission to hospital, hair length, CDT, MCV, GGT, age, number of previous detoxifications, number of previous admissions to hospital, age of first hospitalization, age of onset of disorder, years of duration of disorder and total amount of clomethiazole required for the treatment of withdrawal symptoms.

## DISCUSSION

Numerous tests and devices have been developed and suggested to uncover alcohol consumption (Gill and Soyka, 1997; Laposata, 1999). Biological tests can, in addition to self reports, provide clinicians, forensic toxicologists, judges, counsellors and programme evaluators etc. with complementary information. Other roles of biomarkers in alcoholism treatment have been recently discussed by Allen and Litten (2001). These include serving as outcome variables in treatment efficacy studies, early identification of relapse in patients in abstinence-oriented interventions, and serving as a basis for feedback to enhance patient motivation for change. Granted their ability to monitor heavy drinking use over long periods of time, FAEE hair levels may play an important and unique role in both clinical practice and research. The present study is, to our knowledge, the first to compare  $C_{FAEE}$  in hair with self reports and biomarkers including GGT, MCV, and PEth (Varga *et al.*, 1998, 2000; Wurst *et al.*, 2003) as measures of ethanol intake exceeding 50 g/day for the previous period of at least 2 weeks.

ROC curve analysis indicated a sensitivity for  $C_{FAEE}$  of 100% and a specificity of 90% at a cut-off of 0.29 and sensitivity of 94.4% and a specificity of 90%, respectively, for a cut-off of 0.46 ng/mg in distinguishing teetotallers/social drinkers and heavy drinkers/alcohol dependency (AUC = 0.983). In contrast, only 55.6% of alcohol-dependent patients with previous chronic ethanol intake were identified with the cut-off of 1.0 ng/mg.

As in previous investigations, concentrations of FAEE were also found in hair of teetotallers (Auwärter *et al.*, 2001), but  $C_{FAEE}$  was never above 0.4 ng/mg. The origin of FAEE in these cases is not yet clear. Generally social drinkers have  $C_{FAEE} < 0.4$  ng/mg, but there were also some cases of social drinkers with  $C_{FAEE}$  up to 0.8 ng/mg. However, the possibility can not be dismissed that some of those individuals, although currently being non-dependent social drinkers, drank at levels comparable to those of alcohol-dependent patients. Furthermore, it is seen from the cases described in this study, as well as from the patients on withdrawal treatment described previously (Auwärter *et al.*, 2001), that a cut-off of 1.0 ng/mg leads to excessive numbers of negative results. In contrast to

the post-mortem cases with clearly higher  $C_{FAEE}$ , the patients investigated here during inpatient detoxification washed their hair regularly, thus decreasing FAEE incorporation from sebum.

Interesting as well and meriting further research attention is the near-comparable performance of ethyl palmitate in isolation to  $C_{FAEE}$  in this study. Nevertheless,  $C_{FAEE}$  is the more usual criterion for ethanol consumption and additionally, possible external contamination can more likely be excluded by the ratio of the four esters.

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